ACTA
STOMATOLOGICA
CROATICA
www.ascro.hr

Acta stomatol Croat. 2013;47(1):10-20.
IZVORNI ZNANSTVENI RAD
ORIGINAL SCIENTIFIC PAPER

Ana Badovinac[1], Genadij Razdorov[2], Lovorka Grgurević[2], Ivan Puhar[1], Darije Plančak[1], Darko Božić[1]

Primjena tehnologije LC-MS/MS za proteomsku analizu gingivnog tkiva: eksperimentalno istraživanje

The Application of LC-MS/MS Technology for Proteomic Analysis of Gingival Tissue: a Pilot Study

[1] Zavod za parodontologiju Stomatološkog fakulteta Sveučilišta u Zagrebu i Klinički zavod za parodontologiju Kliničkog bolničkog centra Zagreb, Zagreb, Hrvatska
Department of Periodontology, School of Dental Medicine, Zagreb, and Clinical Department of Periodontology, Clinical Hospital Center Zagreb, Zagreb, Croatia
[2] Centar za translacijska i klinička istraživanja Medicinskog fakulteta Sveučilišta u Zagrebu, Hrvatska
Center for Translational and Clinical Research, School of Medicine, Zagreb, Croatia

Sažetak
Svrha: Željelo se pronaći optimalne uvjete i metode za identifikaciju maksimalnog broja proteina u uzorcima tkiva gingive pacijentice s agresivnim parodontitisom. Pritom smo se koristili tehnologijom *Label-free* kvantifikacije, tekućinske kromatografije i spektrometrije masa (LC –MS/MS), te usporedili proteom zdravih i bolesnih uzoraka tkiva gingive. **Materijali i metode:** Četiri uzorka gingivnog tkiva (dva zdrava i dva bolesna) uzeta su od pacijentice, inače nepušačice, koja boluje od teškog oblika generaliziranog agresivnog parodontitisa. Proteini tkivnog lizata razdvojeni su 1D gel-elektroforezom nakon koje je slijedila digestija u gelu te mjerenje proteina nanoljestvicom HPLC-sistema preko nanoelektro spreja, ionizacijskog izvora i spektrometra mase. Dobiveni podaci obrađeni su u programu MaxQuant. **Rezultati:** *Label-free* kvantifikacija i LC-MS/MS analiza, zajedno s pripremom uzoraka, 1D gel-elektroforezom i digestijom u gelu pokazali su se kao učinkovita metoda za analizu proteoma gingivnog tkiva. Ukupan broj identificiranih i kvantificiranih proteina iznosio je 2429. Proteini koji su pokazali pojačanu ekspresiju u tkivu zahvaćenom agresivnim parodontitisom bili su: imunoglobulinski gama-1 lanac regije C, imunoglobulinski kapa lanac regije C i imunoglobulinski gama-3 lanac regije C (*Ig gamma-1 chain C region, Ig kappa chain C region i Ig gamma-3 chain C region)*, a desmoplakin, aneksini, proteini S100-A8/A9 i keratini pokazali su sniženu ekspresiju. **Zaključak:** Ovo eksperimentalno istraživanje omogućuje novi uvid u proteomski profil zdravog i bolesnog gingivnog tkiva, što bi moglo pridonijeti napretku u dijagnostici i prognozi te boljem razumijevanju patogeneze agresivnog parodontitisa. Tehnologija LC-MS/MS pokazala se učinkovitom kad je riječ o proteomskoj analizi gingivnog tkiva.

Zaprimljen: 29. siječnja 2013.
Prihvaćen: 3. ožujka 2013.

Adresa za dopisivanje
Ana Badovinac, dr. med. dent.
i doc. dr. sc. Darko Božić
Sveučilište u Zagrebu
Stomatološki fakultet
Zavod za parodontologiju
Gundulićeva 5, HR – 10000 Zagreb,
Hrvatska
Tel: +3851489920
badovinac@sfzg.hr, bozic@sfzg.hr

Ključne riječi
parodontitis, agresivni; gingiva; proteomika; spektrometrija masa; biopsija; bjelančevine vezane na mikrotubule; imunoglobulin kappa lanci; dezmoplakini; aneksini; keratini; S100 bjelančevine

Uvod

Parodontitis je jedna od najčešćih bolesti usne šupljine, a karakterizira ga upala gingive i resorpcija potporne kosti (1). Bakterijska infekcija oralnog biofilma aktivira upalni proces te prirođeni i stečeni imunosni odgovor, što rezultira destrukcijom parodontnih tkiva. Budući da su upalni i imunosni odgovor glavni u razvoju parodontnih bolesti, znanje o čimbenicima domaćina važno je za razumijevanje patogeneze parodontnih bolesti (2).

Parodont se sastoji od četiriju glavnih komponenti – gingive, parodontnog ligamenta, cementa i potporne kosti. Spojni epitel nalazi se između gingivnog sulkusa te mekog i mineraliziranog vezivnog tkiva parodonta štiteći tako tkiva iznad kojih se nalazi, a ujedno kontrolira i stalni mikrobni izazov u gingivnom sulkusu. Zbog takve pozicije spojni epitel igra važnu ulogu u homeostazi tkiva i obrani od mikroorganizama i njihovih produkata (3). U nekim slučajevima

Introduction

Periodontitis is one of the most common oral diseases characterized by gingival inflammation and resorption of the alveolar bone (1). Infection with oral biofilm bacteria activates inflammatory and host immune responses, innate and adaptive, which lead to the destruction of periodontal tissues. Since inflammatory and immune responses play a crucial role in the development of periodontal diseases, the knowledge of the host related factors is vital for the understanding of the pathogenesis of periodontal diseases (2).

The periodontium consists of four main components: gingiva, periodontal ligament, cementum and the alveolar bone. The junctional epithelium, situated between the gingival sulcus and periodontal soft and mineralized connective tissue, protects the underlying tissues and controls the constant microbial challenge found in the gingival sulcus. Based on its position, this epithelium plays an important role in tissue ho-

obrambeni odgovor domaćina nije dovoljan, pa se pojavljuju upalne lezije u gingivi i nastaje destrukcija vezivnog tkiva, a pretvorba spojnog epitela u epitel džepa smatra se zaštitnim znakom razvoja parodontitisa (4). U većini radova u kojima se istražuje parodontitis znanstvenici su se fokusirali na ograničeni broj proteina te je zbog toga bilo nemoguće istražiti složenost posrednika koji sudjeluju u lokalnom imunosnom odgovoru lezije zahvaćene parodontitisom (5).

Parodontitis se povezuje s nekoliko sistemskih bolesti i stanja, primjerice s kardiovaskularnim bolestima, aterosklerozom, dijabetesom, respiratornim bolestima i komplikacijama tijekom trudnoće (6, 7). Posljednjih nekoliko godina u istraživanju sistemske biologije zdravlja i bolesti sve važnije postaju proteomske tehnologije (8). Budući da u patološkim promjenama koje se zbivaju tijekom parodontitisa postoji razlika u ekspresiji mnogih molekula, iscrpne analize ekspresije proteina u odabranim uzorcima otvaraju nove znanstvene mogućnosti koje bi mogle obogatiti dosadašnje znanje o proteinima uključenima u patogenezu parodontitisa (9). Biološke tekućine, kao primjerice plazma i urin, bile su proteomski istraživane, a u parodontologiji su se proteomske analize rabile za proučavanje proteoma cementoblasta, sulkusne tekućine (GCF-a) i sline zdravih i bolesnih ispitanika (9 –16).

Bostanci i suradnici analizirali su GCF kako bi identificirali i kvantificirali proteine zdravih ispitanika i pacijenata s agresivnim parodontitisom (17). Ukupno su identificirana i kvantificirana 154 proteina, a autori također zaključuju da bi se proteinski sastav GCF-a mogao iskoristiti kao pomoćno sredstvo u dijagnostici parodontne bolesti. GCF je bio i predmet istraživanja Ngoa i suradnika (13). Njihova je namjera bila identificirati peptide i proteine GCF-a na mjestima sa znakovima upale kod pacijenata s kroničnim parodontitisom u fazi održavanja. Identificirano je 66 proteina te je istaknuto kako bi bolje razumijevanje GCF-a moglo pomoći u daljnjim spoznajama o patogenezi parodontne bolesti. Promjene proteina GCF-a tijekom upalnih procesa istraživali su Grant i njegovi kolege na modelu eksperimentalnog gingivitisa (9). Identificirana su 202 proteina, neki od njih bili su već prije poznati, a neki tek identificirani otkrili su strukturalne komponente gingive koje još nisu bile zabilježene. Mnogo posla obavljeno je i u području proteomskih analiza sline. Upotreba različitih tehnologija za proteomska istraživanja u laboratorijima diljem svijeta rezultirala je katalogom od 2290 proteina koji se nalaze u slini (18).

Sve te analize rezultirale su identifikacijom novih molekula uključenih u patogenezu parodontitisa i uvelike pridonijele novim spoznajama u parodontologiji – možda je čak pronađen put kako se mogu otkriti biomarkeri na kojima posljednjih desetljeća intenzivno rade mnogi znanstvenici (19, 20). Proteomika je značajno poboljšala znanje o GCF-u i slini, no do danas ne postoje podaci od proteomskoj analizi parodontnog tkiva pacijenata s agresivnim parodontitisom. Svrha ovog istraživanja bila je pronaći optimalne uvjete metode za identifikaciju proteina u uzorcima tkiva gingive pacijentice s agresivnim parodontitisom koristeći se tehnologijom *Label-free* kvantifikacije, tekućinske kromatografije i spektrometrije mase (LC – MS/MS), te usporediti proteom zdravih i bolesnih uzoraka gingivnog tkiva.

meostasis and defense against microorganisms and their products (3). In some cases the host defense system is not sufficient which leads to the formation of inflammatory lesions in the gingiva followed by connective tissue breakdown. The conversion of the junctional epithelium to the pocket epithelium is considered as a hallmark in the development of periodontitis (4). Most studies investigating periodontitis have focused only on a limited number of proteins and are thus unable to examine the complexity of mediators that participate in the local immune response of the periodontitis lesion (5).

Periodontitis has been linked to several systemic diseases such as cardiovascular diseases, atherosclerosis, diabetes mellitus, respiratory diseases and unfavorable pregnancy outcomes (6, 7). During the last few years, proteomic technologies have become an important tool for the exploration of system biology in health and disease (8). Since there is a change in the expression of plethora of molecules during the pathological processes that occur in periodontitis, proteome research, which allows comprehensive analyses of expressed proteins in a selected sample, thus opening new scientific opportunities that could enhance our knowledge of proteins that are involved in the pathogenesis of periodontitis (9). Human biological fluids such as plasma and urine have been investigated utilizing the proteomic approach, while in periodontology, proteomic analyses were used to investigate the proteomes of cementoblasts, gingival crevicular fluid (GCF) and saliva within healthy or diseased subjects (9-16).

Bostanci et al. performed analysis of the GCF exudate to identify and quantify proteins within healthy individuals and patients suffering from aggressive periodontitis (17). A total of 154 proteins were identified and quantified, and the authors concluded that the protein content of GCF could potentially be used as a diagnostic aid in periodontal disease. GCF was also a subject of the research for Ngo et al. (13). Their aim was to identify peptides and proteins found in GCF from inflamed sites from subjects with chronic periodontitis that were currently in the maintenance phase of the treatment. In total, 66 proteins were identified pointing out that understanding of GCF may help in further understanding of the pathogenesis of periodontal disease. Temporal changes of proteins in GCF during the inflammatory process in an experimental gingivitis model were investigated by Grant et al. (9). They identified 202 proteins, some of them were already known, but some newly identified proteins highlighted structural components of the gingiva that have not been seen before. A lot of work has also been done in the proteomic analysis of whole saliva. Usage of different proteomic technologies by many laboratories around the world resulted with a catalogue of 2290 identified proteins in whole saliva (18).

All of these analyses greatly contributed to new knowledge, identifying novel molecules involved in the pathogenesis of periodontitis, and could potentially lead to the discovery of disease biomarkers which have been a subject of interest to many researchers working on periodontal diseases during the last decades (19, 20). Proteomics has significantly increased our knowledge of the gingival cervicular fluid (GCF) and saliva, however, to date, there are still no proteomic analyses of periodontal tissues in patients with aggres-

Materijali i metode

Uzimanje uzoraka gingivnog tkiva

Uzorci tkiva gingive uzeti su od pacijentice nepušačice u dobi od 28 godina koja boluje od teškog oblika generaliziranog agresivnog parodontitisa, a inače je zdrava. Agresivni parodontitis dijagnosticiran je na temelju kriterija Američke akademije za parodontologiju (AAP-a) (21), a istraživanje je odobrilo Etičko povjerenstvo Stomatološkog fakulteta u Zagrebu. Prije početka inicijalne parodontološke terapije na Zavodu za parodontologiju Stomatološkog fakulteta u Zagrebu pacijentica je potpisala informirani pristanak, a zatim su uzeti uzorci gingivnog tkiva – dva uzorka s mjesta s velikom dubinom sondiranja i teškim gubitkom kosti, te dva bez znakova parodontne bolesti i rendgenski vidljivog gubitka kosti. Nakon lokalne anestezije uzorci gingive uzeti su s bolesnih i zdravih mjesta i to iz interproksimalnog područja, a uključivali su vezivno tkivo i epitel. Karakteristike mjesta biopsije pokazane su u tablici 1. Uzorci su isprani fiziološkom otopinom kako bi se uklonili tragovi krvi te su nakon toga stavljeni u epruvete volumena 2 mL (Eppendorf, Njemačka) s 2 mL stabilizacijskog reagensa RNAlater (Qiagen, SAD) u skladu s uputama proizvođača. Prvu noć bili su pohranjeni na temperaturi od 4° C, a nakon toga na -20° C do sljedećih analiza.

sive periodontitis. The aim of this pilot study was to find an optimal method conditions to identify proteins from human gingival tissue samples in a patient with aggressive periodontitis using label-free quantitative liquid chromatography mass spectrometry technology (LC-MS/MS), and to compare the proteome of healthy and diseased gingival tissue samples.

Materials and methods

Gingival tissue samples

Gingival tissue samples were obtained from a nonsmoking female patient, 28 years of age, suffering from severe generalized aggressive periodontitis, who was otherwise healthy. Aggressive periodontitis was diagnosed based on the criteria of the American Academy of Periodontology (21), and the study was approved by the Ethics Committee at the School of Dental Medicine in Zagreb. Written informed consent was signed by the patient before the beginning of the initial periodontal therapy at the Department of Periodontology, School of Dental Medicine in Zagreb. Prior to the beginning of the initial periodontal therapy, four gingival tissue samples were obtained from the patient. Two samples were taken from deep sites with severe bone loss and two from sites with no signs of periodontal disease and radiographic bone loss. Following local anesthesia, gingival samples were harvested from the interproximal area, and also included the connective tissue and the epithelium of the diseased and healthy sites. The characteristics of biopsied sites are shown in Table 1. Samples were first rinsed with sterile saline solution to remove all blood and then stored in 2 mL tubes (Eppendorf, Germany) with 2mL of RNAlater stabilization reagent (Qiagen, USA) according to the manufacturer's instructions. First the samples were stored overnight at 4°C, and then transferred at -20°C till further analysis.

Tablica 1. Parodontni indeksi mjesta biopsije
Table 1 Periodontal indices of biopsied sites

	Bolesna mjesta • Diseased sites		Zdrava mjesta • Healthy sites	
Mjesto biopsije • Biopsy site	44 distalno 44 distally	26 distalno 26 distally	45 distalno 45 distally	35 distalno 35 distally
O'Leary plak indeks (PI) • O'Leary Plaque Index (PI)	-	+	-	-
Krvarenje pri sondiranju (BOP) • Bleeding on probing (BOP)	+	+	-	-
Dubina sondiranja (PPD) • Pocket probing depth (PPD)	8 mm	10 mm	2 mm	2 mm
Recesija (REC) • Recession (REC)	4 mm	4 mm	1 mm	0 mm
Klinički gubitak pričvrstka (CAL) • Clinical attachment loss (CAL)	12 mm	14 mm	-	-

Natrijev dodecil-sulfatni poliakrilamid gel-elektroforeza (SDS – PAGE) i digestija u gelu

Uzorci su izvađeni iz stabilizacijskog reagensa RNAlater te su izvagani. Po dva uzorka svakog stanja spojena su u jedan uzorak, zatim su izrezani na manje komade, smrznuti tekućim dušikom i usitnjeni do praškaste konzistencije. Pufer za lizu stanica (4-postotni natrijev dodecil-sulfat (SDS), 0,1 M-dithiotretiol u 0,1 M Tris-HCl, pH 7,6) dodan je usitnje-

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel digestion

Samples were removed from RNAlater and weighed. Two samples for each condition were pooled, cut into small pieces, frozen with liquid nitrogen and minced to a powder consistency. Lysis buffer (4% sodium dodecyl sulfate (SDS), 0.1 M dithiothretiol in 0.1 M Tris-HCl, pH 7.6) was added to minced samples at constant buffer to tissue ratio, heated at

nim uzorcima u istom odnosu praška prema tkivu, deset je minuta grijan na 96° C i centrifugiran 15 minuta na 12000x g. Centrifuga supernatanta je ponovljena, ali ovaj put 30 minuta. Ukupna koncentracija proteina u uzorcima procijenjena je prema UV-metodi na 280 nm. Doprinos nukleinskih kiselina umanjen je korištenjem apsorpcije na 260 nm (1,55 A280 – 0,76 A260 = ukupno proteina mg/mL) (22). Uzorci su izjednačeni potpuno čistom vodom do jednakih proteinskih koncentracija i pomiješani s SDS-PAGE puferom uzorka 4 x (Life Technologies, SAD).

Proteini lizata odvojeni su SDS-PAGE-om rabeći Nu-PAGE 4 – 12-postotni Bis-Tris gel (Life Technologies, SAD) prema uputama proizvođača, te su nakon toga obojeni Simply Blue Safe Stainom (Life Technologies, SAD). Nakon odbojavanja u vodi i 12-postotnom natrijevu kloridu (NaCl) svaka vrpca gela izrezana je na šest dijelova koji su zatim usitnjeni na još manje komade. Digestija u gelu radila se prema protokolu Shevchenka i suradnika (23). Ukratko, proteini su reducirani 10 mM dithiothreitolom (DTT) i zatim alkilirani 55 mM jodoacetamidom (IAA). Digestija proteina tripsinom (13 ng/µL u 50 mM amonijeva bikarbonata) trajala je preko noći. Triptički peptidi ekstrahirani su iz komadića gela postupnom inkubacijom u 30-postotnom acetonitrilu (ACN), 0,5-postotnoj octenoj kiselini (CH3COOH) i nakon toga u B-otopini (80% ACN, 0,5% CH3COOH), te na kraju u čistom ACN-u. ACN je uparen iz ekstrahiranih peptida centrifugiranjem u vakuumu. Poslije digestije u gelu slijedilo je koncentriranje i mikro-pročišćavanje peptida s pomoću *stop and go* ekstracijskih tipsova (Stage Tips) prema Rappsliberu i suradnicima (24). Ukratko, *stage tipsovi* napravljeni su od ekstrakcijskih diskova C18 Empore (3M, SAD), namočeni metanolom i uravnoteženi otopinom A (0,5% CH3COOH). Ekstrahirani peptidi zakiseljeni su solucijom A i naneseni na uravnotežene *tipsove.* Soli su isprane otopinom A, te su peptidi eluirani s *tipsova* otopinom B. Na kraju je ACN uparen s odsoljenih i pročišćenih peptida centrifugiranjem u vakuumu.

Tekućinska kromatografija – spektrometrija mase (LC – MS/MS)

Peptidi su razdvojeni i nakon toga izmjereni sistemom HPLC nanoljestvice Easy-nLC (Proxeon Biosystems) vezane za spektrometar mase LTQ-Orbitrap Discovery (Thermo Scientific) kroz nanoelektrosprejni ionizacijski izvor (Proxeon Biosystems) (25). Ukratko, peptidi su naneseni na nanokolonu C18 kućne izrade napravljene pakiranjem *picofrit* kapilare (New Objective, promjera 75 µm i promjera vrha od 10 µm) s kašom od Luna 3 µm C18(2) faze (Phenomenex) u metanolu. Nakon toga su peptidi uklonjeni iz kolone primjenom linearnog gradijenta otopine B u otopini A (od 3 % do 35 % B otopine tijekom 4,5 sati). MS je mjerio peptide u orbitrapnom analizatoru (10^6 iona pri rezoluciji 30000) i istodobno top 20 peptida fragmentirano u linearnoj ionskoj stupini (3000 iona) koristeći se dinamičkim isključenjem kako bi se spriječilo ponavljanje fragmentacije istaknutih peptida.

Analiza podataka

Neobrađeni podaci uneseni su i obrađeni u programu MaxQuant prema opisu Coxa i Manna (26). Kompletan

96°C for 10 minutes and clarified by centrifugation for 15 minutes at 12000x g. Centrifugation of supernatant was repeated for 30 minutes. Total protein concentration in samples was estimated by UV method at 280nm. Nucleic acid contribution was subtracted using absorption at 260nm (1.55 A280 – 0.76 A260 = total proteins mg/mL) (22). Samples were equalized by ultra pure water to the same total protein content and were mixed with SDS-PAGE sample buffer 4x (Life Technologies, USA).

Proteins from the tissue lysate were separated by SDS-PAGE using Nu-PAGE 4-12% Bis-Tris gel (Life Technologies, USA) as instructed by the manufacturer, and subsequently stained with a Simply Blue Safe Stain (Life Technologies, USA). After destaining in water and in 12% NaCl, each gel lane was cut into 6 slices and then into smaller pieces. In-gel digestion was done according to the protocol described by Shevchenko et al. (23). Shortly, proteins were reduced by 10 mM dithiothreitol (DTT), and alkylated by 55 mM iodoacetamide (IAA). Proteins were over night digested by trypsin (13 ng/µL in 50 mM ammonium bicarbonate). Tryptic peptides were extracted from gel pieces by stepwise incubation in 30% acetonitrile (ACN), 0.5% acetic acid (CH3COOH); then in solution B (80% ACN, 0.5% CH3COOH) and then in pure ACN. ACN was evaporated from pulled extracted peptides by vacuum centrifugation. After in-gel digestion procedure, peptides were concentrated and micro-purified with the stop-and go extraction tips (StageTips) described by Rappsliber et al. (24). Briefly, StageTips were made from C18 Empore extraction disk (3M, SAD) as described. Tips were wetted by methanol and equilibrated by solution A (0.5% CH3COOH). Extracted peptides were acidified by solution A, and loaded onto equilibrated tips. Salts were washed by solution A, and peptides were eluted from tips by solution B. ACN was evaporated from desalted and purified peptides by vacuum centrifugation.

Liquid Chromatography – Mass Spectrometry (LC-MS/MS) analysis

Peptides were separated and then measured by nanoscale HPLC system Easy-nLC (Proxeon Biosystems) coupled to an LTQ-Orbitrap Discovery mass spectrometer (Thermo Scientific) through a nano-electrospray ionization source (Proxeon Biosystems) (25). Briefly, peptides were loaded onto C18 nano column, made in house by slurry packing Picofrit capillary (NewObjective, 75 µm fused silica diameter, 10 µm tip diameter) with Luna 3 µm C18(2) material (Phenomenex). Peptides were eluted from column by linear gradient of solution B in solution A (from 3% B to 35% B in 4.5 hours), and electrosprayed directly into mass spectrometer. Mass spectrometer was measuring peptides in orbitrap analyzer (10^6 ions at 30000 resolution setting) and, in parallel, top. 20 peptides were fragmented in linear ion trap (3000 ions), using dynamic exclusion to prevent recurring fragmentation of prominent peptides.

Data Analysis

Raw data were processed by MaxQuant software as described by Cox and Mann (26). Uniprot Human complete

uniprotov humani proteomski set bio je pretražen koristeći se postavljenim parametrima MaxQuanta. Svim identificiranim proteinima dodijeljen je uniprotov pristupni broj. Genska ontologija obavljena je u bioinformatičkom programu DAVID (27).

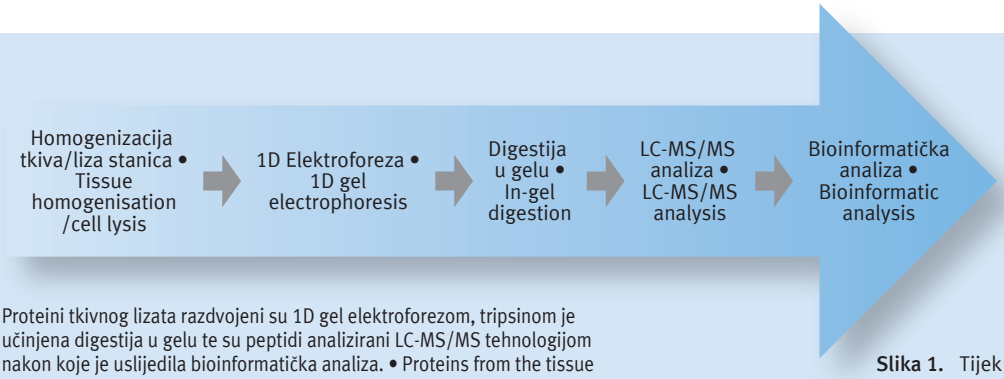
Rezultati

Label-free kvantitativna LC – MS/MS analiza rabila se za karakterizaciju proteina u uzorcima gingivnog tkiva zdravih mjesta i mjesta zahvaćenih parodontnom bolešću kod pacijentice s teškim oblikom generaliziranog agresivnog parodontitisa. Takav pristup, zajedno s pripremom uzoraka, 1D gel-elektroforezom i digestijom proteina u gelu, pokazao se kao učinkovita metoda za analizu proteoma epitelnog i vezivnog tkiva parodonta (slika 1.).

proteome set was searched using default MaxQuant parameters. All identified proteins are referred to by Uniprot accession number. Gene Ontology (GO) was performed using the DAVID bioinformatic software (27).

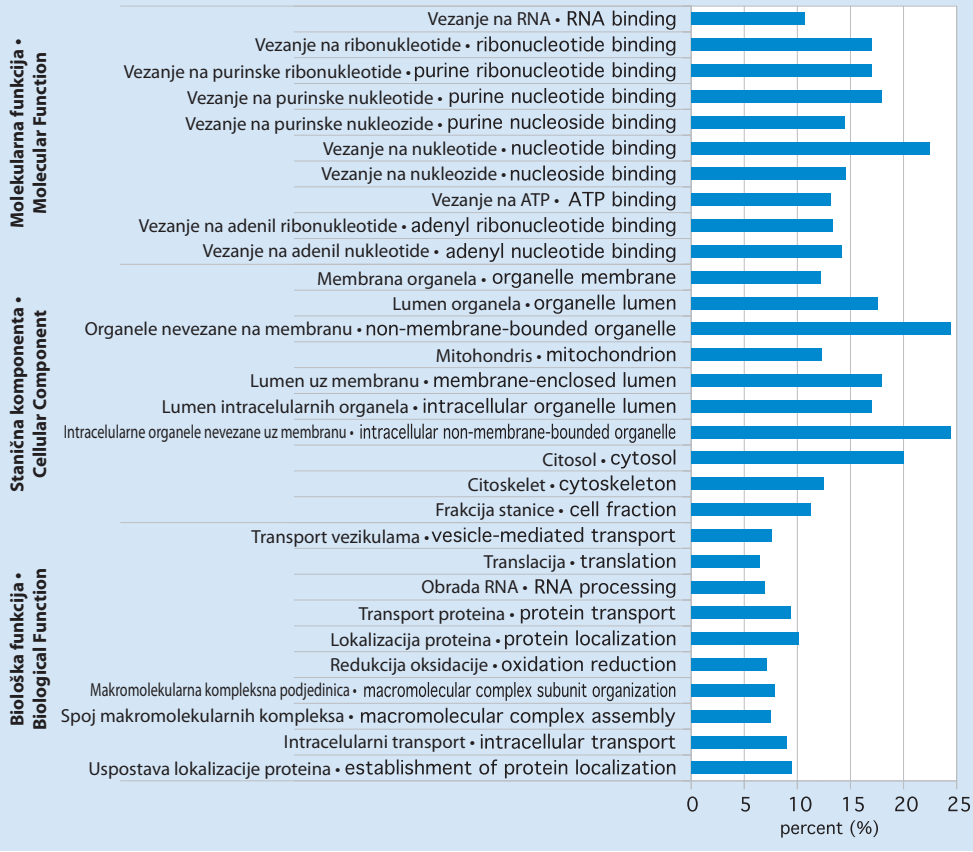
Results

Label-free quantitative LC-MS/MS analysis was used to characterize proteins in tissue samples collected from healthy sites and sites with signs of periodontal disease in a patient with a severe form of generalized aggressive periodontitis. This approach, together with sample preparation, 1D gel electrophoresis and in-gel digestion of proteins proved to be an effective method for the proteome analysis of the epithelial and connective periodontal tissues (Figure 1).



Proteini tkivnog lizata razdvojeni su 1D gel elektroforezom, tripsinom je učinjena digestija u gelu te su peptidi analizirani LC-MS/MS tehnologijom nakon koje je uslijedila bioinformatička analiza. • Proteins from the tissue lysate were separated by 1D gel electrophoresis, digested using trypsin, and analyzed by LC-MS/MS technology, followed by bioinformatic analysis.

Slika 1. Tijek eksperimentalnih procedura.
Figure 1 Workflow of experimental procedures.



Slika 2. Analiza genske ontologije (GO) proteomski identificiranih proteina. Prikazano je deset najistaknutijih grupa za svaki od 3 kriterija GO.
Figure 2 Gene ontology (GO) analysis of proteins identified by proteomics. Ten most prominent groups per each of three different GO criteria are shown.

Ukupan broj identificiranih i kvantificiranih proteina u spojenim uzorcima iznosio je 2429. U tablici 2. zabilježeno je 60 proteina koji su pokazali najveće razlike u ekspresiji između zdravih i bolesnih uzoraka. Među identificiranim proteinima s jačom ekspresijom u bolesnom negoli u zdravom uzorku, našli su se: imunoglobulinski gama-1 lanac regije C (P01857), imunoglobulinski kapa lanac regije C (P01834), imunoglobulinski gama-3 lanac regije C (P1860), imunoglobulinski lambda-2 lanac regije C (P0CG05), imunoglobulinski gama-2 lanac regije C (P01859) i protein *versican core* (P13611) . Proteini koji su, u usporedbi sa zdravim uzorkom, pokazali slabiju ekspresiju u bolesnom uzorku, bili su: desmoplakin (P15924), spojeni plakoglobin (P14923), plakofilin-1 (Q13835), desmoglein-1 (Q02413), aneksin A1 (P04083), protein S100-A9 (P06702), toplinsko stresni protein beta-1 (P04792), protein S100-A8 (P05109), elongacijski faktor 1-alfa 1 (P68104), alfa enolaza (P06733), keratini tipa I i II, te prolargin (P51888).

Genska ontologija (GO) pokazuje distribuciju 2429 identificiranih proteina prema njihovoj molekularnoj funkciji, staničnoj komponenti i biološkom procesu (slika 2.). Nukleotidima vezani proteini bili su najzastupljenija skupina proteina prema kriteriju molekularne funkcije. Prema kriteriju stanične komponente, organeli bez membrane i unutarstanični organeli bez membrane pokazali su se kao dvije najzastupljenije skupine proteina. I na kraju proteini zaduženi za lokaciju i pronalaženje te lokacije bile su dvije najzastupljenije skupine prema kriteriju biološke funkcije.

A total of 2429 proteins were identified and quantified in two pooled tissue samples. Table 2 presents 60 most prominent proteins differently expressed in the healthy and diseased sample. Among the identified proteins that were upregulated in the diseased sample compared to the healthy sample were: Ig gamma-1 chain C region (P01857), Ig kappa chain C region (P01834), Ig gamma-3 chain C region (P01860), Ig lambda-2 chain C regions (P0CG05), Ig gamma-2 chain C region (P01859), and Versican core protein (P13611). Proteins that were identified in the diseased sample and downregulated when compared to the healthy sample were: Desmoplakin (P15924), Junction plakoglobin (P14923), Plakophilin-1 (Q13835), Desmoglein-1 (Q02413), Annexin A1 (P04083), Protein S100-A9 (P06702), Heat shock protein beta-1 (P04792), Protein S100-A8 (P05109), Elongation factor 1-alpha 1 (P68104), Alpha-enolase (P06733), Keratins type I and II, and Prolargin (P51888).

The GO shows distribution of 2429 proteins identified in our study according to their molecular function, cellular compartment and biological process (Figure 2). Nucleotide binding proteins were the most abundant group by the molecular function criteria. By cellular component criteria, non-membrane-bound organelle and intracellular non-membrane-bound organelle were the two most abundant groups of proteins. Finally, by the biological function, proteins in charge of protein localization and establishment of protein localization were the two most abundant groups.

Tablica 2. 60 proteina koji su pokazali najveće razlike u ekspresiji između zdravih i bolesnih uzoraka. Proteini su izabrani prema ukupnom intenzitetu i promjeni ekspresije kada se bolesni uzorak tkiva usporedio sa zdravim.
Table 2 60 most prominent proteins differently expressed in the healthy and diseased sample. Proteins are selected based on their total intensity and expression level shift in diseased tissue sample when compared to a healthy one.

MaxQuant id	Uniprot pristupni broj • Uniprot Accession Number	Ime proteina • Protein name	Broj jedinstvenih peptida • No. of unique peptides	Pokrivenost sekvence (%)• Sequence Coverage (%)	PEP	Regulacija • Regulation
112	A8K2U0	Alpha-2-macroglobulin-like protein 1	38	33.4	1.84E-268	↓
879	E9PBV3	Suprabasin	19	58.5	0	↓
1433	O60437	Periplakin	93	50.7	0	↓
1560	P01009	Alpha-1-antitrypsin	31	69.4	0	↑
1303	P01023	Alpha-2-macroglobulin	41	34.3	0	↑
1562	P01024	Complement C3	97	58.1	0	↑
1612	P01834	Ig kappa chain C region	9	89.6	0	↑
1613	P01857	Ig gamma-1 chain C region	8	32.1	0	↑
1614	P01859	Ig gamma-2 chain C region	7	31.3	0	↑
1615	P01860	Ig gamma-3 chain C region	5	24.9	0	↑
1622	P02452	Collagen alpha-1(I) chain	29	27	0	↑
1624	P02545	Prelamin-A/C	55	63	0	↓
1642	P02787	Serotransferrin	50	61.5	0	↑
1655	P04083	Annexin A1	27	67.3	0	↓
1665	P04264	Keratin, type II cytoskeletal 1	36	58.1	0	↓
1676	P04792	Heat shock protein beta-1	20	90.7	2.16E-303	↓
1685	P05109	Protein S100-A8	11	60.2	4.61E-111	↓
1710	P06702	Protein S100-A9	10	82.5	1.50E-189	↓
1714	P06733	Alpha-enolase	25	53.2	0	↓
1731	P07355	Isoform 2 of Annexin A2	32	74.2	0	↓

MaxQuant id	Uniprot pristupni broj • Uniprot Accession Number	Ime proteina • Protein name	Broj jedinstvenih peptida • No. of unique peptides	Pokrivenost sekvence (%)• Sequence Coverage (%)	PEP	Regulacija • Regulation
1095	P08123	Collagen, type I, alpha 2	27	24.7	6.28E-289	↑
98	P08133	Annexin A6	42	55.2	0	↑
1748	P08238	Heat shock protein HSP 90-beta	24	31.4	0	↓
1759	P08670	Vimentin	47	73.2	0	↑
1782	P0CG05	Ig lambda-2 chain C regions	5	57.5	0	↑
1795	P11021	78 kDa glucose-regulated protein	37	54.6	0	↑
1797	P11142	Heat shock cognate 71 kDa protein	22	38.5	0	↓
1814	P12035	Keratin, type II cytoskeletal 3	23	48.6	0	↓
1815	P12109	Collagen alpha-1(VI) chain	41	41.2	0	↓
1816	P12110	Collagen alpha-2(VI) chain	31	29.8	0	↓
1817	P12111	Collagen alpha-3(VI) chain	6	2.5	0	↓
1834	P13611	Versican core protein	3	0.8	0	↑
1837	P13639	Elongation factor 2	41	51	0	↓
654	P13645	Keratin, type I cytoskeletal 10	29	49.9	0	↓
1855	P14625	Endoplasmin	39	41.3	0	↑
1859	P14923	Junction plakoglobin	19	35.8	0	↓
1870	P15924	Desmoplakin	265	71.3	0	↓
1912	P19012	Keratin, type I cytoskeletal 15	1	3.3	0	↓
658	P19013	Keratin, type II cytoskeletal 4	32	44.9	0	↓
1935	P21333	Filamin-A	112	54.3	0	↑
2066	P31947	14-3-3 protein sigma	16	74.2	0	↓
2071	P32926	Desmoglein-3	28	36.9	0	↓
2174	P47929	Galectin-7	9	85.3	4.25E-159	↓
2232	P51888	Prolargin	21	54.5	5.38E-277	↓
2276	P58107	Epiplakin	14	16.3	0	↓
2335	P62805	Histone H4	11	54.4	8.30E-191	↑
2351	P63104	14-3-3 protein zeta/delta	17	62.4	0	↓
2361	P68104	Elongation factor 1-alpha 1	21	50.6	0	↓
2366	P68871	Hemoglobin subunit beta	11	68.7	0	↑
2368	P69905	Hemoglobin subunit alpha	4	21.8	0	↑
2385	P84243	Histone H3.3	1	23.5	4.24E-138	↑
2413	Q02413	Desmoglein-1	30	39.1	0	↓
2431	Q05707	Collagen alpha-1(XIV) chain	67	45.3	0	↑
2448	Q08188	Protein-glutamine gamma-glutamyltransferase E	30	52.1	0	↓
2521	Q13835	Plakophilin-1	38	59.7	0	↓
2530	Q14134	Tripartite motif-containing protein 29	32	47.1	0	↓
2580	Q15149	Isoform 4 of Plectin	2	0.5	0	↓
681	Q61782	Type I epidermal keratin	2	21.5	0	↓
2935	Q92817	Envoplakin	94	48.1	0	↓
723	Q99715	Collagen alpha-1(XII) chain	158	53.4	0	↑

PEP - posterior error probability
↓ - sniženi u bolesnom uzorku • downregulated in diseased tissue sample
↑ - povišeni u bolesnom uzorku • upregulated in diseased tissue sample

Rasprava

Proteomsko istraživanje omogućuje kvalitativne i kvantitativne analize razlika između proteoma različitih stanja, uključujući različite genotipe, bolesti i utjecaj lijekova (28, 29). U dosadašnjim proteomskim istraživanjima parodontnih bolesti znanstvenici su analizirali GCF ili slinu kako bi uočili razlike između zdravog i bolesnog parodonta (10), identificirali moguće biomarkere (11) ili pak promjene vezane uz aktivnost same bolesti (30). No, do danas ne postoji proteomsko istraživanje gingivnog tkiva zdravog i bolesnog parodonta kod pacijenata s agresivnim parodontitisom. U našem istraživanju uspoređeni su proteomi gingivnog tkiva s mjesta s teškim gubitkom kosti i s onoga bez znakova parodontne bolesti. Koliko znamo ovo je prvo istraživanje koje se koristilo tehnologijom *Label-free* LC-MS/MS za identifikaciju i kvantifikaciju proteina zdravih i bolesnih uzoraka gingive osobe s agresivnim parodontitisom.

Među najistaknutijim identificiranim proteinima u uzorcima imunoglobulini (Ig) su pokazali razlike u ekspresiji. Ig su proteini koje proizvode B-limfociti, a imunolosni sustav koristi se njima za identifikaciju i neutralizaciju stranih tvari, poput bakterija i virusa. Obrambeni mehanizmi uz pomoć B-limfocita uključuju njihovu pretvorbu u plazma-stanice i stvaranje imunoglobulina koji su sposobni identificirati i vezati se za antigen (31). Prema riječima Berghlundha i Donatija, udjel B-limfocita u parodontnoj leziji je oko 18 posto, što ih svrstava na drugo mjesto najzastupljenijih stanica, odmah iza plazma-stanica koje čine oko 50 posto stanica parodontne lezije (32). Budući da B-limfociti stvaraju imunoglobulin, naše istraživanje također ističe njihovu ulogu u patogenezi parodontne bolesti. I istraživanje Bostancija i suradnika pokazalo je da se Ig ubraja među najzastupljenije proteine GCF-a zdravih i parodontitisom zahvaćenih mjesta (17). Baliban i njegovi kolege analizirali su uzorke GCF-a zdravih pojedinaca i osoba s kroničnim parodontitisom te su dobili visoku zastupljenost imunoglobulina i komplementa u obje ispitivane skupine (11). Navedena otkrića potvrđuju važnu ulogu imunoglobulina u lokalnim obrambenim mehanizmima i pokazuju da bi proteomska istraživanja mogla pomoći u daljnjim istraživanjima vezanima za odgovor domaćina u parodontnim bolestima.

U skladu s prijašnjim istraživanjima identificirani su keratini tipa I i II, histoni, desmoplakin, aneksini, proteini S100-A8/A9, elongacijski faktor 1-alfa 1 i mnogi drugi. Među 60 najzastupljenijih proteina u zdravim i bolesnim uzorcima našli su se keratini i članovi porodice proteina koji vežu kalcij, kao što su aneksin A1, A2 (P07355), A6 (P08133) i S100 proteini A8, A9.

S100A8/A9 proteini vežu cink i kalcij te imaju zapaženu ulogu u regulaciji upalnih procesa i imunolosnog odgovora zato što potiču kemotaksiju i adheziju neutrofila (33). Vezanjem kalcija prilagođavaju biološku aktivnost te pokazuju jaku ekspresiju u makrofagima i epitelnim stanicama akutno upaljenih tkiva (34). U istraživanju Haigha i suradnika proteini S100A8/A9 također su pokazali različitu ekspresiju u uzorcima sline u slučaju parodontitisa. Autori su istaknuli upletenost proteina S100 u odgovor domaćina tijekom paro-

Discussion

Proteomic research offers qualitative and quantitative analyses of the differences between proteomes in different conditions, including different genotypes, diseases and drug influences (28, 29). So far, in proteomic studies investigating periodontal diseases, researchers analyzed GCF or saliva to see the differences between healthy and diseased periodontium (10), to identify possible biomarkers (11), and to identify changes associated with the activity of the disease (30). However, to date there is no proteomic research of gingival tissues in healthy and diseased periodontium in patients with aggressive periodontitis. This study compared proteomes of gingival tissues from sites with severe bone loss, and those from sites with no signs of periodontal disease. To the best of our knowledge, this is the first study that used label-free LC-MS/MS technology for the identification and quantification of proteins in healthy and diseased gingival tissue samples in a patient with aggressive periodontitis.

Among the most prominent proteins identified in the tissue samples, immunoglobulins (Ig) were changed in their expression levels. Ig are proteins produced by B cells, used by the immune system to identify and neutralize foreign objects such as bacteria and viruses. Host defense mechanisms of B cells include their transformation into plasma cells and production of Ig that identify and bind to antigens (31). According to Berghlundh and Donati, in periodontitis lesions the portion of B cells is about 18% which puts them in second place among most dominant cell types right after plasma cells which represent about 50% of cells in the periodontitis lesion (32). Since Ig are produced by B cells, our findings further highlight their role in the pathogenesis of periodontal disease. Furthermore, in the study by Bostanci et al., analysis of the GCF exudate from healthy and periodontally diseased sites, also demonstrated that Ig were among the most abundant proteins (17). By analyzing GCF samples from patients with chronic periodontitis and periodontally healthy individuals, Baliban et al. found that Ig and complement were widely detected in both groups (11). These findings confirm the crucial role that Ig may play in the local defense mechanisms, and that proteomic approach could be helpful in further investigations dealing with host response in periodontal diseases.

In accordance with previous reports, Keratins type I and II, Histones, Desmoplakin, Annexins, Protein S100-A8/A9, Elongation factor 1-alpha 1 and many other proteins were identified. In our study, keratins and calcium binding protein family members, such as Annexin A1, A2 (P07355), A6 (P08133) and S100 proteins A8, A9 were among the top 60 prominent proteins in healthy and diseased tissue.

S100A8/A9 are calcium and zinc binding proteins which play a prominent role in the regulation of inflammatory processes and immune response, by inducing neutrophil chemotaxis and adhesion (33). They modulate biological activity via calcium binding, and they are expressed by macrophages and epithelial cells in acutely inflamed tissues (34). In the study of Haigh et al., S100A8/A9 proteins also showed to be differentially expressed in saliva during periodontitis. The authors highlighted the involvement of S100 proteins in the host response

dontitisa te su ih predložili kao moguće biomarkere za praćenje aktivnosti parodontne bolesti (30).

Aneksini su skupina proteina koji vežu kalcij i povezani su s upalnim i obrambenim odgovorom (35). Iako se povezuju s upalnim procesima, istraživanja Bostancija i suradnika te Grant i njezinih kolega pokazala su da bi aneksini A1 i A2 mogli biti povezani s parodontnim zdravljem, te su ih predložili za proteine koji održavaju zdravlje parodontnog tkiva (9, 17). Naši rezultati slažu se s rezultatima navedenih istraživanja za aneksine A1 i A2, no za razliku od tih istraživanja mi smo identificirali i aneksin A6 koji je pokazao jaču ekspresiju u bolesnim uzorcima. Aneksin A6 impliciran je u posredovanje agregacije endosoma i fuzije vezikula epitela tijekom egzocitoze. Temeljem navedenoga jasno je da su potrebna daljnja istraživanja kako bi se dokumentirala i objasnila uloga aneksina u parodontnoj bolesti.

Naše istraživanje otkrilo je pojačanu ekspresiju histona H4 (P62805) i histona H3,3 (P84243) u bolesnim uzorcima. Histoni su jezgreni proteini važni u mnogobrojnim biološkim procesima, primjerice u apoptozi, antimikrobnom djelovanju, organizaciji strukture DNK, regulaciji transkripcije, popravcima i replikaciji DNK te u stabilizaciji kromosoma. Njihove povišene razine zabilježene su u serumu osoba s traumatskim ozljedama i sepsom (36), a u proteomskom istraživanju eksperimentalnog gingivitisa imali su slabiju ekspresiju pri kraju rezolucijskog razdoblja (9). Pojačana ekspresija histona u bolesnim uzorcima našeg istraživanja mogla bi upućivati na povezanost tih proteina s bakterijskom aktivnošću, te su svakako potrebna daljnja istraživanja kako bi se objasnila njihova uloga u parodontitisu.

Među najzastupljenijim proteinima sa sniženom ekspresijom u bolesnim uzorcima bili su desmoplakin i keratini tipa I i II. Niža ekspresija keratina mogla bi značiti slabiju pregradnju i diferencijaciju epitela u tkivu gingive zahvaćenom parodontnom bolešću. Taj se nalaz slaže s nalazom istraživanja Bostancija i suradnika (17) te potvrđuje dobro poznatu činjenicu da je brzina pregradnje zdravog sulkularnog epitela jedna od najbržih pregradnji epitelnog tkiva općenito (37).

Desmoplakin, protein dezmosoma, uključen je u organizaciju dezmosomskog kadherin-plakoglobinskog kompleksa i u sidrenje srednjih filamenata u dezmosome (38). Taj protein bio je među proteinima snižene ekspresije u bolesnim uzorcima, upućujući na to da je njegova ekspresija u dezmosomima epitela džepa snižena. Razlog za to mogao bi biti podatak da gubitak staničnog kontinuiteta spojnog epitela pridonosi stvaranju džepa u trenutku kada se stanice epitela odvoje od površine zuba (3, 39). Povećanje broja mononuklearnih i polinuklearnih leukocita također pridonosi fokalnoj dezintegraciji spojnog epitela (40), permeabilnost spojnog epitela mijenja se i sve to završava pojačanom filtracijom GCF-a koja je čest klinički znak ranog gingivitisa.

Kao i ostala proteomska istraživanja ni naše nije identificiralo citokine za koje se zna da sudjeluju u patogenezi parodontne bolesti (9, 11, 41). Razlog je vjerojatno u činjenici da su citokini prisutni u rasponu od pikograma u mililitru i da ih zaklanjaju veći proteini koji se nalaze u mikrogramima u mililitru kao, primjerice, albumin. Kako bi se prevladali ti problemi identifikacije proteina, potrebne su nove metode za

during periodontitis, and suggested them as new potential biomarkers for monitoring periodontal disease activity (30).

Annexins are a group of calcium binding proteins that are associated with inflammatory and defense response (35). Although these proteins are related with the inflammatory process, studies by Bostanci et al. and Grant et al. found that Annexin A1 and A2 are more likely to be associated with periodontal health suggesting a possible role of these proteins in maintaining periodontal health (9, 17). For Annexin A1 and A2, the results from our study were in accordance with the above mentioned studies, but unlike Bostanci and Grant, we have also identified Annexin A6 which was upregulated in the diseased samples. Annexin A6 has been implicated in mediating the endosome aggregation and vesicle fusion in secreting epithelia during exocytosis. Further studies are needed to document and explain the role of Annexin protein family members in periodontal disease.

Our data also revealed upregulation of Histone H4 (P62805) and Histone H3.3 (P84243). Histones are nuclear proteins that play a role in multiple biological processes such as apoptosis, antimicrobial action and DNA structure organization. Furthermore, histones play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. Presence of elevated levels of histones in serum has been reported in traumatic injury and sepsis (36). Histones have been identified in proteomic studies of experimental gingivitis showing downregulation at the end of the resolution period (9). The upregulation of histones in the diseased samples could point to their important role in bactericidal activity. Future studies are needed to elucidate the role of histones in periodontitis.

Among the most abundantly downregulated proteins in the diseased samples were Desmoplakin, and Keratins type I and II. Lower expressions of Keratins could indicate that the turnover and differentiation of oral epithelia is decreased in the gingival tissue affected by periodontal disease. This finding is in agreement with a study of Bostanci et al. (17), and confirms the well known fact that the turnover rate of healthy sulcular epithelium is known to be the one of the most rapid of all the epithelial tissues (37).

Desmoplakin, protein of desmosomes, is involved in the organization of the desmosomal cadherin-plakoglobin complexes and in the anchoring of intermediate filaments to the desmosomes (38). This protein was shown to be among the most downregulated proteins in disease, implying that the expression of desmoplakin in desmosomes is reduced in the pocket epithelium. This may be due to the fact that loss of cellular continuity of the junctional epithelium attributes to a pocket formation when epithelial cells detach from the tooth surface (3, 39). Increased number of mononuclear and polymorphonuclear leukocytes also contributes to the focal disintegration of the junctional epithelium (40), and the permeability of the junctional epithelium is altered leading to increase in GCF filtration which is a common clinical sign of early gingivitis.

Similar to other studies using the proteomic approach, we were unable to detect cytokines that are known to be involved in the pathogenesis of periodontal disease (9, 11, 41). The reason for this could be that these cytokines are usually present in

pripremu uzoraka i prikupljanje podataka te novi analitički postupci koji se koriste analitičkim algoritmima potrebnima za poticanje otkrivanja proteina i pretvorbu detektiranih signala u identificiranu molekulu. Osim toga, korištenjem sadašnjih tehnologija za analizu većeg broja uzoraka trebat će mjeseci ili godine kako bi se diferencirali proteini vezani za bolesti od varijacija humanih uzoraka (42, 43).

Zaključak

Ovo eksperimentalno istraživanje prvo je istraživanje proteoma gingivnog tkiva i daje nam novi pogled na proteinski profil zdravog i bolesnog gingivnog tkiva. Nove spoznaje mogle bi pridonijeti poboljšanju dijagnostike, prognostike i boljem razumijevanju patogeneze parodontitisa. Tehnologija LC-MS/MS pokazala se kao učinkovita metoda za proteomsku analizu gingivnog tkiva. Iako se u ovom istraživanju uspoređuju dva uzorka jednog pacijenta, rezultati pokazuju da se ekspresija proteina gingivnog tkiva zahvaćenog agresivnim parodontitisom značajno razlikuje od ekspresije proteina klinički zdravog gingivnog tkiva te da se ističu neke skupine proteina, kao što su imunoglobulini, keratini, proteini koji vežu kalcij i ostali koji još nisu povezani s parodontitisom. U našem laboratoriju trenutačno još traje istraživanje na većem broju uzoraka, a zadak je karakterizirati i usporediti proteome gingivnog tkiva zdravih i agresivnim parodontitisom zahvaćenih mjesta.

Zahvala

Rad je rađen u sklopu projekata 065-0650444-0415 (Sistemni aspekti u etiologiji parodontnih bolesti – voditelj Darije Plančak) Ministarstva znanosti, obrazovanja i sporta Republike Hrvatske.

Izjava

Autori ističu da nisu bili ni u kakvom sukobu interesa.

picograms/milliliter range and are overshadowed by larger proteins that are present in micrograms/milliliter such as albumin. To overcome such identification problems, we need new sample preparation methods, data acquisition and analysis steps using new data analysis algorithms which are needed to enhance protein discovery and turn the detected signals into molecular identification. Furthermore, using the current technologies, analyzing many samples will take months or years in order to differentiate diseases related proteins from background variations in human samples which are enormous (42, 43).

Conclusion

This pilot study is the first study of gingival tissue proteome providing a novel insight into the protein profiles of gingival healthy and diseased tissues, which may contribute to the improvements in diagnosis, prognosis and better understanding of the pathogenesis of periodontitis. LC-MS/MS technology showed to be an effective method for the proteome analysis of the gingival tissues. Although this is a pilot study with one patient that compared two pooled samples, the results revealed that protein expression in gingival tissues affected by aggressive periodontitis significantly differs from the protein expression in clinically healthy gingival tissues, pointing out to some protein groups such as: immunoglobulins, keratins, calcium binding protein family members and others previously not associated with periodontitis. Larger sample size investigations are currently underway in our laboratory in order to characterize and compare gingival tissue proteome in healthy and diseased sites in patients with aggressive periodontitis.

Acknowledgements

This paper was supported by the Ministry of Science, Education and Sport of the Republic of Croatia Grant No. 065-0650444-0415 (chief investigator Darije Plančak, project "Systemic aspects in the etiology of periodontal diseases").

Transparency declaration

The authors deny any conflicts of interest.

Abstract

Aim: The aim of this study was to find an optimal method to identify maximal number of proteins from human gingival tissue samples in a patient with aggressive periodontitis using label-free quantitative liquid chromatography mass spectrometry technology (LC-MS/MS), and to compare proteome of healthy and diseased gingival tissue samples. **Materials and methods:** Four gingival tissue samples (2 healthy, 2 diseased) were obtained from a nonsmoking female patient suffering from severe generalized aggressive periodontitis. Proteins from the tissue lysate were separated by 1D gel electrophoresis, in gel digested, measured by nano-scale HPLC system coupled to a mass spectrometer through a nano-electrospray ionization source. Raw data were processed by MaxQuant software. **Results:** Label-free quantitative LC-MS/MS analysis together with sample preparation, 1D gel electrophoresis and in-gel digestion showed to be an effective method for the proteome analysis of the gingival periodontal tissues. A total of 2429 proteins were identified and quantified. Among proteins upregulated in disease were: Ig gamma-1 chain C region, Ig kappa chain C region, Ig gamma-3 chain C region, and among downregulated proteins in the disease were: Desmoplakin, annexins, S100-A8/A9 proteins, and keratins. **Conclusions:** This pilot study is providing a novel insight into the protein profiles of gingival healthy and diseased tissues, which may contribute to the improvements in diagnosis, prognosis and better understanding of aggressive periodontitis pathogenesis. LC-MS/MS technology proved to be an effective method for the proteome analysis of the gingival tissues.

Received: January 29, 2013
Accepted: March 5, 2013

Address for correspondence
Ana Badovinac, DMD
University of Zagreb
School of Dental Medicine
Department of Periodontology
Gundulićeva 5
HR-10000 Zagreb, Croatia
Tel: +38514899202
badovinac@sfzg.hr; bozic@sfzg.hr

Key words
Aggressive periodontitis; Gingiva; Proteomics; Mass Spectrometry; Biopsy; Microtubule-Associated Proteins; Immunoglobulin kappa-Chains; Antigens, CD3; Desmoplakins; Annexins; Keratins; S100 Proteins

References

1. Savage A, Eaton KA, Moles DR, Needleman I. A systematic review of definitions of periodontitis and methods that have been used to identify this disease. J Clin Periodontol. 2009 Jun;36(6):458-67.
2. Taubman MA, Valverde P, Han X, Kawai T. Immune response: the key to bone resorption in periodontal disease. J Periodontol. 2005 Nov;76(11 Suppl):2033-41.
3. Schroeder HE, Listgarten MA. The gingival tissues: the architecture of periodontal protection. Periodontol 2000. 1997 Feb;13:91-120.
4. Schroeder HE, Listgarten MA. The junctional epithelium: from strength to defense. J Dent Res. 2003 Mar;82(3):158-61.
5. Buduneli N, Kinane DF. Host-derived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. J Clin Periodontol. 2011 Mar;38 Suppl 11:85-105.
6. Puhar I, Lovrencic-Huzjan A, Sodec-Simicevic D, Strineka M, Bozic D, Plancak D. Carotid Intima-Media Thickness in Patients with Chronic and Aggressive Periodontitis. Acta Stomatol Croat. 2012;46(4):255-62.
7. Bosnjak A, Plancak D, Curilovic Z. Advances in the Relationship between Periodontitis and Systemic Disease. Acta Stomatol Croat. 2001;35(2):259-71.
8. Hu S, Loo JA, Wong DT. Human body fluid proteome analysis. Proteomics. 2006 Dec;6(23):6326-53.
9. Grant MM, Creese AJ, Barr G, Ling MR, Scott AE, Matthews JB, et al. Proteomic analysis of a noninvasive human model of acute inflammation and its resolution: the twenty-one day gingivitis model. J Proteome Res. 2010 Sep 3;9(9):4732-44.
10. Wu Y, Shu R, Luo LJ, Ge LH, Xie YF. Initial comparison of proteomic profiles of whole unstimulated saliva obtained from generalized aggressive periodontitis patients and healthy control subjects. J Periodontal Res. 2009 Oct;44(5):636-44.
11. Baliban RC, Sakellari D, Li Z, DiMaggio PA, Garcia BA, Floudas CA. Novel protein identification methods for biomarker discovery via a proteomic analysis of periodontally healthy and diseased gingival crevicular fluid samples. J Clin Periodontol. 2012 Mar;39(3):203-12.
12. Carneiro LG, Venuleo C, Oppenheim FG, Salih E. Proteome data set of human gingival crevicular fluid from healthy periodontium sites by multidimensional protein separation and mass spectrometry. J Periodontal Res. 2012 Apr;47(2):248-62.
13. Ngo LH, Veith PD, Chen YY, Chen D, Darby IB, Reynolds EC. Mass spectrometric analyses of peptides and proteins in human gingival crevicular fluid. J Proteome Res. 2010 Apr 5;9(4):1683-93.
14. Grgurevic L, Macek B, Durdevic D, Vukicevic S. Detection of bone and cartilage-related proteins in plasma of patients with a bone fracture using liquid chromatography-mass spectrometry. Int Orthop. 2007 Dec;31(6):743-51.
15. Grgurevic L, Macek B, Erjavec I, Mann M, Vukicevic S. Urine release of systemically administered bone morphogenetic protein hybrid molecule. J Nephrol. 2007 May-Jun;20(3):311-9.
16. Bozic D, Grgurevic L, Erjavec I, Brkljacic J, Orlic I, Razdorov G, et al. The proteome and gene expression profile of cementoblastic cells treated by bone morphogenetic protein-7 in vitro. J Clin Periodontol. 2012 Jan;39(1):80-90.
17. Bostanci N, Heywood W, Mills K, Parkar M, Nibali L, Donos N. Application of label-free absolute quantitative proteomics in human gingival crevicular fluid by LC/MS E (gingival exudatome). J Proteome Res. 2010 May 7;9(5):2191-9.
18. Loo JA, Yan W, Ramachandran P, Wong DT. Comparative human salivary and plasma proteomes. J Dent Res. 2010 Oct;89(10):1016-23.
19. Champagne CM, Buchanan W, Reddy MS, Preisser JS, Beck JD, Offenbacher S. Potential for gingival crevice fluid measures as predictors of risk for periodontal diseases. Periodontol 2000. 2003;31:167-80.
20. Loos BG, Tjoa S. Host-derived diagnostic markers for periodontitis: do they exist in gingival crevice fluid? Periodontol 2000. 2005;39:53-72.
21. Armitage GC. Development of a classification system for periodontal diseases and conditions. Ann Periodontol. 1999 Dec;4(1):1-6.
22. Aitken A, Learmonth MP. Protein Determination by UV Absorption. In: Walker JM, editor. The Protein Protocols Handbook. 2nd ed: Humana Press; 2002. p. 3-6.
23. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 2006;1(6):2856-60.
24. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc. 2007;2(8):1896-906.
25. Gatlin CL, Kleemann GR, Hays LG, Link AJ, Yates JR, 3rd. Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. Anal Biochem. 1998 Oct 1;263(1):93-101.
26. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 2008 Dec;26(12):1367-72.
27. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44-57.
28. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003 Mar 13;422(6928):198-207.
29. Zhu H, Bilgin M, Snyder M. Proteomics. Annu Rev Biochem. 2003;72:783-812.
30. Haigh BJ, Stewart KW, Whelan JR, Barnett MP, Smolenski GA, Wheeler TT. Alterations in the salivary proteome associated with periodontitis. J Clin Periodontol. 2010 Mar;37(3):241-7.
31. Berglundh T, Donati M, Zitzmann N. B cells in periodontitis: friends or enemies? Periodontol 2000. 2007;45:51-66.
32. Berglundh T, Donati M. Aspects of adaptive host response in periodontitis. J Clin Periodontol. 2005;32 Suppl 6:87-107.
33. Gebhardt C, Nemeth J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. Biochem Pharmacol. 2006 Nov 30;72(11):1622-31.
34. Marenholz I, Heizmann CW, Fritz G. S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophys Res Commun. 2004 Oct 1;322(4):1111-22.
35. Perretti M, D'Acquisto F. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. Nat Rev Immunol. 2009 Jan;9(1):62-70.
36. Margraf S, Logters T, Reipen J, Altrichter J, Scholz M, Windolf J. Neutrophil-derived circulating free DNA (cf-DNA/NETs): a potential prognostic marker for posttraumatic development of inflammatory second hit and sepsis. Shock. 2008 Oct;30(4):352-8.
37. Rowat JS, Squier CA. Rates of epithelial cell proliferation in the oral mucosa and skin of the tamarin monkey (Saguinus fuscicollis). J Dent Res. 1986 Nov;65(11):1326-31.
38. Bornslaeger EA, Corcoran CM, Stappenbeck TS, Green KJ. Breaking the connection: displacement of the desmosomal plaque protein desmoplakin from cell-cell interfaces disrupts anchorage of intermediate filament bundles and alters intercellular junction assembly. J Cell Biol. 1996 Aug;134(4):985-1001.
39. Takata T, Donath K. The mechanism of pocket formation. A light microscopic study on undecalcified human material. J Periodontol. 1988 Apr;59(4):215-21.
40. Kowashi Y, Jaccard F, Cimasoni G. Sulcular polymorphonuclear leucocytes and gingival exudate during experimental gingivitis in man. J Periodontal Res. 1980 Mar;15(2):151-8.
41. Bostanci N, Ramberg P, Wahlander A, Grossman J, Jonsson D, Barnes VM, et al. Label-Free Quantitative Proteomics Reveals Differentially Regulated Proteins in Experimental Gingivitis. J Proteome Res. 2013 Jan 4.
42. Veenstra TD. Where are all the biomarkers? Expert Rev Proteomics. 2011 Dec;8(6):681-3.
43. Lubec G, Afjehi-Sadat L. Limitations and pitfalls in protein identification by mass spectrometry. Chem Rev. 2007 Aug;107(8):3568-84.